Insulin Regulation of β-Cell Function Involves a Feedback Loop on SERCA Gene Expression, Ca^{2+} Homeostasis, and Insulin Expression and Secretion†

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ABSTRACT: The insulin receptor signaling pathway is present in β-cells and is believed to be important in β-cell function. We show here that insulin directly regulates β-cell function in isolated rodent islets. Long-term insulin treatment caused a sustained increase in $[Ca^{2+}]_i$ and enhanced glucose-stimulated insulin secretion in rat islets, but failed to increase insulin content. Chronic activation of insulin receptor signaling by IRS-1 overexpression in the β-cell inhibited gene expression of SERCA3, an endoplasmic reticulum Ca^{2+}-ATPase. Insulin gene transcription was stimulated by insulin receptor signaling and insulin mimetic compound (L-783 281) in a glucose- and Grb2-dependent manner. Thus, β-cell SERCA3 is a target for insulin regulation, which implies that β-cell Ca^{2+} homeostasis is regulated in an autocrine feedback loop by insulin. This study identifies a novel regulatory pathway of insulin secretion at the molecular level with two main components: (1) regulation of intracellular Ca^{2+} homeostasis via SERCA3 and (2) regulation of insulin gene expression.

In the early stage of type 2 diabetes or non-insulin-dependent diabetes mellitus, pancreatic β-cells secrete more insulin to compensate for insulin resistance, resulting in hyperinsulinemia. Over the course of the disease, β-cell function progressively deteriorates and eventually fails to compensate for insulin resistance. β-Cell failure is now recognized as a key event for triggering overt diabetes (*1*), although the mechanisms involved are poorly understood. Accumulating data indicate that nutritional and environmental factors, hormonal regulation, and genetic predisposition all affect β-cell function, as reviewed elsewhere (*2*−*9*). Recent studies show that the insulin receptor (IR)[1] signaling system regulates β-cell function, including insulin secretion, insulin and protein biosynthesis, and β-cell growth (*1*, *10*−*17*). The insulin receptor signaling system also differentially affects β-cell function at the level of IR substrates. For example, IRS-2 stimulates β-cell growth (*18*), while IRS-1 affects β-cell Ca^{2+} homeostasis and stimulates insulin secretion but inhibits insulin biosynthesis and β-cell growth (*1*, *11*, *16*, *18*, *19*). Despite these studies, it is still unclear how insulin affects β-cells in the long term and whether hyperinsulinemia plays any role in inducing β-cell dysfunction in type 2 diabetes.

Intracellular Ca^{2+} plays an important role in β-cell function: it is the key intracellular mediator of fuel-stimulated insulin secretion (see reviews in refs *20*−*23*) and directly affects insulin biosynthesis (*24*, *25*). Two sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA2 and SERCA3) have been found in β-cells and play major roles in the sequestration of cytosolic Ca^{2+} into the endoplasmic reticulum (*22*, *23*). Both SERCA2 and SERCA3 gene transcripts have splicing variants resulting in distinct protein isoforms with different tissue distribution and Ca^{2+} affinities (*26*−*30*). Recent studies show that loss of SERCA activities and the reduction of SERCA3 gene expression in β-cells are associated with diabetes in db/db mice (*31*), Goto-Kakizaki Wistar (GK) rats (*32*), and humans (*33*). However, the mechanisms for regulation of SERCA activity and gene expression in the β-cell are yet to be elucidated.

We demonstrate in this study that insulin modulates β-cell Ca^{2+} homeostasis through IRS-1 inhibition of SERCA3 gene expression, revealing a novel pathway for the insulin regulation of β-cell function. We also show that high levels of insulin and glucose stimulate insulin secretion, but fail to increase the intracellular insulin storage capacity, indicating that chronic exposure to insulin and glucose may in the long term impair β-cell function.

EXPERIMENTAL PROCEDURES

Materials. The clonal mouse β-cell line βTC6-F7 and culture conditions were previously described (*10*, *34*). In brief, cells were maintained in high-glucose DMEM (25 mM glucose; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin, and 50 μg/mL streptomycin and incubated at 37 °C in a 10% CO_2/90% air, humidified incubator. L-783 281 was provided by B. Zhang (Merck)

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[1] Abbreviations: ER, endoplasmic reticulum; IR, insulin receptor; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SERCA, sarcoplasmic (endoplasmic) reticulum Ca^{2+}-ATPase.

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and dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution.

β-Cell Culture and Insulin Secretion. Insulin content and secretion assays for cultured β-cells were performed essentially as described previously (*12*, *35*). Total cellular insulin was extracted with acid alcohol. Insulin content was measured by the RIA. The fractional insulin secretion was calculated as the amount of secreted insulin/total insulin content and expressed as a percentage. For insulin secretion in the absence of extracellular Ca^{2+}, Ca^{2+} was omitted and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate (EGTA) (Sigma) was added to chelate all Ca^{2+}. For assessment of amino acid-stimulated insulin release, 10 mM leucine and 5 mM glutamine were used together.

Rat Isolated Islet Preparation and Culture. Islets were isolated from male Sprague-Dawley rats by collagenase P digestion and Ficoll gradient centrifugation as described previously (*36*). The islets were incubated in complete CMRL-1066 (Life Technologies) at 37 °C in 5% CO_2 for 48−72 h under the indicated conditions. The exogenous insulin added to culture media was of bovine origin (Sigma, I-5500). Islets were washed at least four times with Krebs-Ringers' buffer (KRB) [115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 25 mM Hepes (pH 7.4)] without glucose. Twenty islets were subsequently transferred to each glass tube and challenged with 1 mL of KRB with 0 or 20 mM glucose with 1 mM carbachol (CCH, carbamylcholine chloride, Sigma). After incubation at 37 °C in 5% CO_2 for 1 h, the culture supernatant was collected for the insulin assay. Islet insulin content was measured after acid alcohol extraction. The insulin radioimmunoassay was performed using anti-rat insulin antibody and rat insulin standard (Linco, St. Charles, MO).

Cytosolic Free [Ca^{2+}] Measurement. For $[Ca^{2+}]_i$ measurement, β-cells were seeded on microscope glass coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-D-lysine (Sigma) and cultured for 2 days. Cells were incubated in 2 mL of KRB (0.1% BSA) with 2.0 μM fura-2 acetoxy methyl ester (Molecular Probes, Eugene, OR) and 0.2 mg/mL pluronic F-127 (Molecular Probes) at 37 °C for 30 min. The coverslip was then mounted into a perifusion chamber placed on the homeothermic platform of an inverted Zeiss microscope. The cells were perifused with KRB (0.1% BSA) at 37 °C at a flow rate of 1.5 mL/min. The method of $[Ca^{2+}]$ measurement is described in detail elsewhere (*11*). For measuring rat islet $[Ca^{2+}]_i$, isolated islets were cultured under the indicated conditions, then loaded with fura-2 as described above, and placed in a perifusion chamber. The islets were held in position by a glass micropipet and perifused with KRB (0.1% BSA, with the reagents indicated below) at a flow rate of 1.5 mL/min. The perifusion protocol was as follows: 5 min without glucose (G0), 10 min with 20 mM glucose (G20), and 5 min with 30 mM KCl. Two to three islets from each culture condition could be assessed under each condition in each experiment. Data were collected from at least three experiments. $[Ca^{2+}]_i$ in the islets cultured in 5 mM glucose without insulin was set as a control in each experiment. Ca^{2+} concentrations at other conditions were expressed as fold over control to normalize for small instrumental variations in $[Ca^{2+}]$ measurement in different experiments.

Table 1: Media Insulin Levels after Culturing of Rat Islets for 72 h[a]

culture condition	72 h media insulin (nM)	SEM	*n*
5 mM glucose	44.58	7.47	23
5 mM glucose 200 nM insulin	343.70	17.42	16
20 mM glucose	346.56	11.47	17
5 mM glucose 200 nM insulin without islets	248.38	10.91	8
5 mM glucose without islets	<0.1	–	–

[a] Two hundred rat islets were cultured under the indicated conditions in 10 mL of CMRL-1066 media supplemented with 10% fetal bovine serum. Samples of the media were collected after incubation for 72 h, and the insulin concentration was determined by radioimmunoassay.

As indicated in Table 1, insulin concentrations in islet culture media were 346 nM under in vitro "hyperglycemic" conditions (20 mM glucose) and 44 nM at a nonstimulatory glucose concentration (5 mM). Both were higher than rat plasma insulin levels measured in vivo. To mimic the insulin level observed in islets incubated with 20 mM glucose, we experimentally determined that 200 nM insulin was needed. As shown in Table 1, addition of 200 nM exogenous insulin led to a final insulin level of 343 nM at the end of a 72 h incubation which was similar to that caused by 20 mM glucose alone (346 nM). We felt that it was very important to have similar insulin concentrations among the different conditions to draw valid conclusions. We recognize, of course, that these are in vitro conditions. Currently, the precise peri-islet insulin concentration in vivo is unknown. It is believed that insulin levels around insulin-secreting β-cells under physiological conditions in vivo are much higher than that in circulating blood. So although caution should be used to directly apply the in vitro data to in vivo situation, we have successfully demonstrated that insulin influences β-cell Ca^{2+} homeostasis under the conditions described herein (see the Results).

Purification of β-Cell RNA and Reverse Rranscriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was purified from β-cells using the QuickPrep Total RNA Extraction Kit (Pharmacia Biotech, Piscataway, NJ) per the manufacturer's instructions. The first-strand cDNA from 6 μg of total RNA was synthesized using M-MLV Reverse Transcriptase (Life Technology) and Oligo(dT) (Promega) as per the manufacturer's instructions. One-sixth of the cDNA synthesized was subjected to PCR amplification using the Expand High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN). Each PCR mixture also contained the gene specific primers (each at 300 nM) and the mouse β-actin primers (each at 180 nM) (see Table 2 for details), cold dNTPs (each at 0.2 mM), and 4 μCi of [α-^{32}P]dCTP (NEN, Boston, MA). PCR was carried out in a Perkin-Elmer DNA themal Cycler 480 (Perkin-Elmer, Foster City, CA) with the following program: 94 °C for 3 min, followed by 30 cycles (or as indicated) of 94 °C for 1 min, 47 °C for 2 min, and 72 °C for 3 min. The last cycle was followed by a 7 min extension at 72 °C. The linear range of the PCR was between 20 and 35 cycles (data not shown). PCR products were purified by 1.2% agarose gel electrophoresis. Gels were photographed, and the PCR product bands were excised. DNA was then extracted by adding 200 μL of QX1 buffer (solubilization solution) from a QIAGEN gel extraction kit

Table 2: PCR Primers

gene specificity	primer[a]	sequence[b]	T_m (°C)	length of the PCR product (bp)
IRS-1	IRSP4 (F)	5′-CAACAGCAGCAGCAGTCTTC-3′	59.9	370
	IRSP2 (R)	5′-CTGTCCGCATGTCAGCATAG-3′	60.4	
IRS-2	IRS2-EF (F)	5′-CTCAGCCTTCCTCTGCCTCT-3′	50.8	990
	IRS2-FR (R)	5′-CAGGCGGAGACAGACGGGTA-3′	52.8	
SERCA2	mSER2AF (F)	5′-AGTTCATCCGCTACCTCATCTC-3′	51.7	504
	mSER2AR (R)	5′-GGGCATCCTCAGCAAAGACT-3′	53.2	
SERCA2b	mSER2AF (F)	5′-AGTTCATCCGCTACCTCATCTC-3′	51.7	762
	mSER2BR (R)	5′-CGACAGGGAGCAGGAAGAT-3′	51.5	
SERCA3	mSER3AF (F)	5′-GGCCAAGTCAGCAGCAGAG-3′	53.2	401
	mSER3AR (R)	5′-GCGTCATACAGGAACCACCA-3′	53.1	
SERCA3b	mSER3AF (F)	5′-GGCCAAGTCAGCAGCAGAG-3′	53.2	860
	mSER3BR (R)	5′-GTATGGGGTCCTGGAAGTAGTC-3′	50.9	
β-actin	mActinF (F)	5′-CGGCCAGGTCATCACTATT-3′	49.7	326
	mActinR (R)	5′-TGGAAGGTGGACAGTGAGG-3′	50.2	

[a] F, forward primer; R, reverse primer. [b] Primer sequences and locations were based on the following DNA sequences: IRS-1, Genbank accession number L24563; IRS-2, human cDNA, DDBJ/EMBL/Genbank accession number AB000732; SERCA2a and -2b, forward primer (mSER2AF) and 2a reverse primer (mSER2AR) based on mouse SERCA2a cDNA, GenBank accession number AJ223584; SERCA2b, reverse primer (mSER2BR) based on rat SERCA2b (GenBank accession number J04024) and mouse EST sequences (GenBank accession numbers AA832581, AA410070, AI121443, AI121433, AA259320, AA260416, AA432904, AA981304, and AA407618); SERCA3a and -3b, mouse SERCA3 cDNA accession numbers U49394 and U49393, respectively; β-actin, mouse β-actin cDNA, GenBank accession number X03672.

(QIAGENE, Valencia, CA) and incubating at 45 °C for 15 min. The amount of radioactivity incorporated into PCR products was quantified by scintillation counting with 5 mL of Universol scintillation solution (ICN, Costa Mesa, CA). The amounts of amplified SERCA and IRS products were normalized to the amount of β-actin PCR products. Duplicate samples were assayed in each experiment. The data presented herein are from at least four experiments. PCR primer pairs for the specific genes are listed in Table 2. All PCR products were sequenced and compared to the corresponding DNA sequences in the GenBank database (NCBI, Bethesda, MD).

Immunoprecipitation and Western Blotting. Preparation of cell lysates, immunoprecipitation, and Western blotting were performed essentially as described previously (*12*, *35*). Tyrosine-phosphorylated proteins were detected with rabbit polyclonal anti-phosphotyrosine antibody K-18. Anti-Shc antibody was from Transduction (S14630, Lexington, KY). Anti-Grb2 antibody was purchased from Santa Cruz Biotechnology (SC-255, Santa Cruz, CA).

Construction of the Rat Insulin Promoter-Green Fluorescence Protein (RIP-GFP) Reporter Plasmid. The RIP-GFP reporter gene plasmid was constructed as follows. The plasmid RIP tag (*37*) was first digested with *Xba*I, and the resulting DNA ends were filled with the Klenow fragment of DNA polymerase I. The plasmid was subsequently digested with *Bam*HI to obtain the 0.7 kb fragment containing the rat insulin II promoter (RIP). The RIP fragment was then inserted into *Bgl*II−*Sma*I double-digested pEGFP-1 (Promega) to generate the plasmid pRIP-GFP. The control plasmid with constitutive CMV promoter pCMV-GFP was constructed by inserting the 1.1 kb *Bgl*II−*Sma*I CMV promoter fragment from pCI-Neo (Promega) into pEGFP1.

Determination of the Level of Insulin Gene Expression Using the RIP-GFP Reporter Gene Assay. Plasmid DNA was electroporated into β-cells using the following procedure. Cells were trypsinized as described previously (*10*) and resuspended in phosphate-buffered sucrose media at a density of 4×10^6 cells/mL. Electroporation was performed using the Gene Pulser II RF Module (Bio-Rad) with the following parameters: 250 V, 100% modulation, 40 kHz, 6 ms burst duration, and a total of 5 bursts with a 1 s interval. The cells were then placed in six-well plates on glass coverslips and cultured for 20−24 h. Green fluorescence was detected at 507 nm using an excitation wavelength of 488 nm. Insulin gene expression was quantified by measuring the relative intensity of green fluorescence using a Zeiss fluorescence microscope equipped with a cooled CCD camera. Plasmids expressing the wild-type insulin receptor pIRN6, kinase negative insulin receptor pAKN8, and IRS-1 expression vector pCMV-IRS1 were described previously (*10*, *11*). A pcNDA3 vector expressing the IRS-1 mutant defective in Grb2 interaction (IRS-1-R972) was provided by O. Porzio (University of Rome, Rome, Italy). Expression vectors for a constitutive active form of PI-3K (p110*) and a kinase deficient mutant of PI-3K (p110ΔKin) (*38*, *39*) were all kindly provided by M. Birnbaum (University of Pennsylvania). IRS-2 expression plasmid pCMV-IRS2 was obtained from T. Gustafson (Metabolex, Hayward, CA). SHC plasmids Rldn-SHC and Rldn-3F-SHC and the control plasmid Rldn vector were kindly provided by T. Sasaoka (Toyama, Japan).

Data Analysis. Data were analyzed by a paired *t* test, one-way or two-way ANOVA using PRISM 3.0 (GraphPad Software, San Diego, CA); a p of ≤ 0.05 was considered significant.

RESULTS

Activation of insulin receptor signaling by overexpression of either the insulin receptor (IR) or IRS-1 stimulates insulin secretion and increases free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) levels in β-cells (*10*, *11*). Short-term insulin stimulation (30 s) has been shown to evoke a rise in $[Ca^{2+}]_i$ and to initiate insulin secretion in pancreatic islet β-cells (*17*). To investigate the chronic effect of insulin on β-cells further, we isolated rat islets and cultured them in 200 nM insulin and 5 mM (nonstimulatory) or 20 mM (stimulatory) glucose for 72 h. Addition of 200 nM insulin and 20 mM glucose resulted in similar actual insulin concentrations in the culture media (Table 1). The cytosolic $[Ca^{2+}]$ of isolated islets was measured with the perifusion protocol described in Experi-

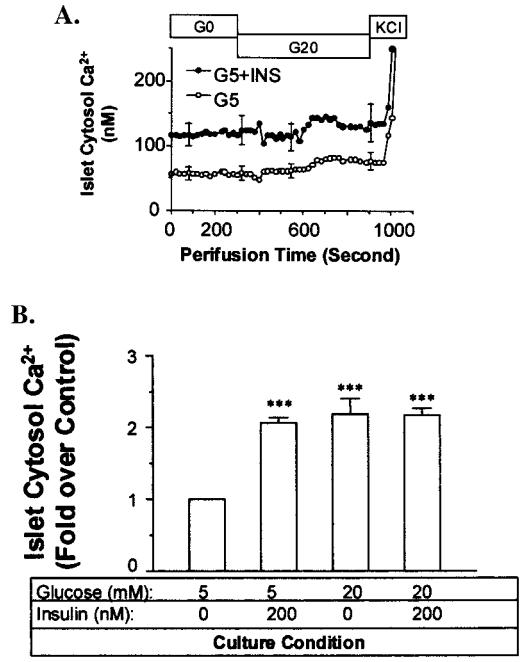

FIGURE 1: Insulin increases the cytosolic [Ca^{2+}] in isolated rat islets. Isolated islets were incubated for 72 h with insulin or glucose, washed, and perifused as described in Experimental Procedures. (A) Time course of the cytosolic [Ca^{2+}] after acute stimulation. Culture conditions were as follows: G5 and G5+INS, islets precultured in 5 mM glucose (○) and 5 mM glucose and 200 nM insulin (●), respectively. Perifusion conditions are indicated at the top of the curves: G0 and G20, 0 and 20 mM glucose, respectively, with 30 mM KCl. (B) Effect of insulin on basal cytosolic [Ca^{2+}]. Cytosolic Ca^{2+} concentrations of islets precultured under different conditions were measured in the absence of glucose (G0). The cytosolic [Ca^{2+}] of the islets precultured in 5 mM glucose without insulin was set as a control. Data were obtained from three experiments with at least six islets measured. Three asterisks indicate that $P < 0.0001$.

mental Procedures. Islets precultured in 200 nM insulin exhibited a significant 2.1-fold increase in basal level $[Ca^{2+}]_i$ compared to control islets (Figure 1A). Rat islets precultured in 20 mM glucose or 20 mM glucose and 200 nM insulin exhibited an increase in basal $[Ca^{2+}]_i$ similar to that of the islets cultured in 200 nM insulin (Figure 1B). These results provide the first evidence that long-term (3 day) exposure of rat islets to a high level of insulin causes sustained elevation of islet $[Ca^{2+}]_i$ even in the absence of glucose.

We also assessed insulin's long-term effect on insulin secretion and production. After treatment for 3 days with 200 nM insulin, rat islets exhibited a 1.4-fold increase in the level of glucose-stimulated insulin secretion (Figure 2A). Pretreatment with 20 mM glucose resulted in a similar increase [1.6-fold over the control (Figure 2A)]. The 3 day insulin treatment did not stimulate insulin production, while 20 mM glucose increased the level of islet insulin production by 2.2-fold (Figure 2B). This differential effect between insulin and glucose was also reflected in the islet insulin content. The 72 h glucose treatment significantly reduced the islet insulin content by 48%, while insulin caused no reduction (Figure 2C). Fractional insulin secretion (amount of secreted insulin/intracellular insulin content) reflects the β-cell insulin secretory capacity. Long-term insulin treatment significantly increased the islet fractional insulin secretion by 1.6-fold in the presence of 20 mM glucose. Pretreatment with glucose increased the fractional insulin secretion at both 0 mM (2.8-fold) and 20 mM glucose (3.5-fold) (Figure 2D).

Our previous study had demonstrated that the insulin receptor and IRS-1 are involved in regulating β-cell insulin secretion and Ca^{2+} homeostasis (*10*, *11*). Chronic overexpression of IRS-1 has been shown to inhibit endoplasmic reticulum (ER) Ca^{2+} uptake in β-cells possibly via modulation of ER Ca^{2+}-ATPase (SERCAs) activity. IRS-1 inhibition

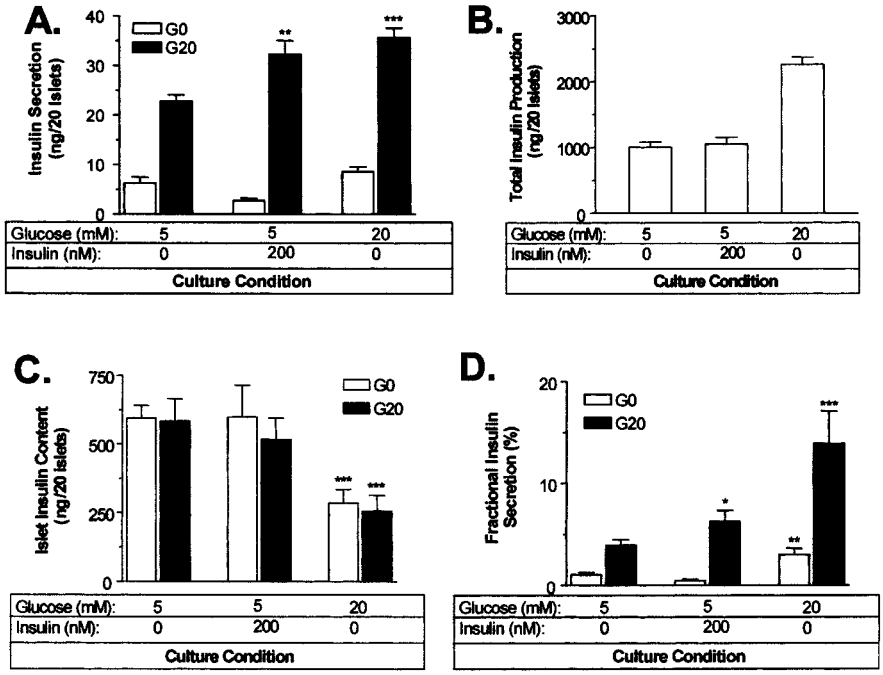

FIGURE 2: Rat islet function after insulin and glucose 72 h pretreatment. Rat islets were cultured in 200 nM insulin with 5 or 20 mM glucose for 3 days as indicated below each graph. The islets were then washed and assayed for (A) insulin secretion; (B) insulin production, e.g., total insulin amount secreted into the media and islet insulin content; (C) insulin content; and (D) fractional insulin secretion (amount of secreted insulin/total insulin content). Assay conditions are indicated in Experimental Procedures and in each graph: G0 and G20, 0 and 20 mM glucose, respectively. One asterisk indicates that $p < 0.05$; two asterisks indicate that $p < 0.001$ and three asterisks $p < 0.0001$.

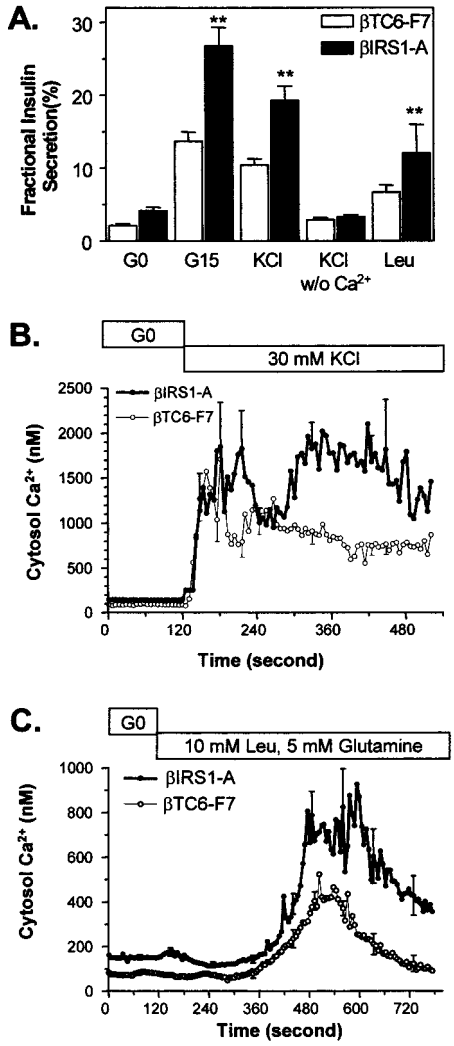

FIGURE 3: Effect of IRS-1 overexpression on insulin secretion and intracellular free $[Ca^{2+}]$ in β-cells. (A) Fractional insulin secretion: white bars, parental cell βTC6-F7; and black bars, βTC6-F7 cells overexpressing IRS-1 (βIRS1-A). Conditions are as follows: G0 and G15, 0 and 15 mM glucose, respectively; with 30 mM KCl, KCl without Ca^{2+}, and 30 mM KCl without calcium but with 1 mM EGTA; 10 mM leucine. Two asterisks indicate that $p < 0.001$. (B and C) Time course of cytosolic free $[Ca^{2+}]$. Cells were perifused with G0 (without glucose) KRB and 30 mM KCl or 10 mM leucine as indicated. Every data point on the curves represents the mean value of at least 30 cells from three experiments. Only representative standard errors of the mean (SEM) are shown. Ca^{2+} concentrations in IRS-1-overexpressing cells (βIRS1-A, ●) were significantly higher than the control (βTC6-F7, ○).

of ER Ca^{2+} uptake increased $[Ca^{2+}]_i$ and enhanced glucose-stimulated fractional insulin secretion (*11*). We have previously generated β-cell lines that overexpress IRS-1 by 2-fold, and shown that a 2-fold overexpression of IRS-1 was sufficient to increase fractional insulin secretion (*11*). Overexpression of IRS-1 also enhances potassium chloride (KCl)- and leucine-stimulated fractional insulin secretion (Figure 3A). In addition, potassium and leucine treatment also significantly increased $[Ca^{2+}]_i$ in β-cells overexpressing IRS-1 (Figure 3B,C). These results show that β-cell insulin secretion capacity is closely linked to $[Ca^{2+}]_i$. IRS-1 modulation of β-cell Ca^{2+} homeostasis affects insulin secretion stimulated by glucose as previously shown and also leucine-induced and depolarization-induced insulin secretion.

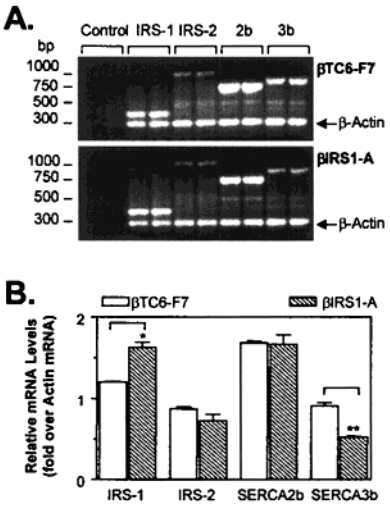

FIGURE 4: IRS-1 overexpression decreases the level of SERCA3b expression in β-cells. mRNA levels were quantitated by RT-PCR as described in Experimental Procedures. (A) Representative agarose gels showing the RT-PCR products from the βTC6-F7 cells (upper gel) and from the IRS-1-overexpressing βIRS1-A cells (lower gel). The primers that were used are described in Table 2. Control, RNA samples were amplified directly with PCR without reverse transcription; 2b and 3b represent SERCA2b and -3b, respectively. The bands for β-actin are indicated to the right of the gels. Quantitative results are shown in panel B. One asterisk indicates that $p = 0.02$; two asterisks indicate that $p = 0.007$. Data were obtained from four experiments each performed in duplicate.

To understand the molecular mechanism of IRS-1 modulation on β-cell Ca^{2+} homeostasis, we examined the effect of IRS-1 overexpression on β-cell gene expression using reverse transcriptase-polymerase chain reaction (RT-PCR). The IRS-1 mRNA level was higher in the IRS-1-overexpressing βIRS1-A cells than in the control as expected (Figure 4). Transcripts of two ER Ca^{2+}-ATPases (SERCA2 and SERCA3) were detected in insulinoma βTC6-F7 cells. We determined that IRS-1 overexpression specifically induced a 42% inhibition of SERCA3 gene expression (Figure 4). Expression levels of other genes such as IRS-2 and SERCA2 were not affected by IRS-1 overexpression. Both the SERCA2 and SERCA3 gene transcripts have splicing isoforms *a* and *b* which differ in tissue distribution and Ca^{2+} affinities (*26*−*30*). Overexpression of IRS-1 in β-cells did not change the ratio of splicing variants for both SERCA2 and SERCA3. More than 90% of the SERCA2 gene transcripts and ∼70% of the SERCA3 mRNA were in the *b* isoform in both the control and IRS-1-overexpressing β-cells (data not shown).

Next we examined the role of insulin receptor signaling in regulating insulin gene expression. We identified two more insulin receptor signaling molecules in β-cells: Shc, another insulin receptor substrate (*40*), and Grb2, a key adapter protein for insulin receptor substrates (*41*, *42*) (Figure 5A). Using reciprocal immunoprecipitation analysis, we demonstrated that β-cell Grb2 binds to tyrosine-phosphorylated IRS-1 in an insulin-dependent manner (Figure 5A, lanes 1 and 2). The association between Grb2 and Shc in β-cells seemed to be insulin-independent (Figure 5A, lanes 1, 2, and 5−10). Grb2 predominantly associated with the tyrosine-phosphorylated 52 kDa form of the Shc proteins (P-Shc, Figure 5A, lanes 1, 2, and 8−10). Less than 50% of the tyrosine-phosphorylated Shc protein was bound to Grb2 in

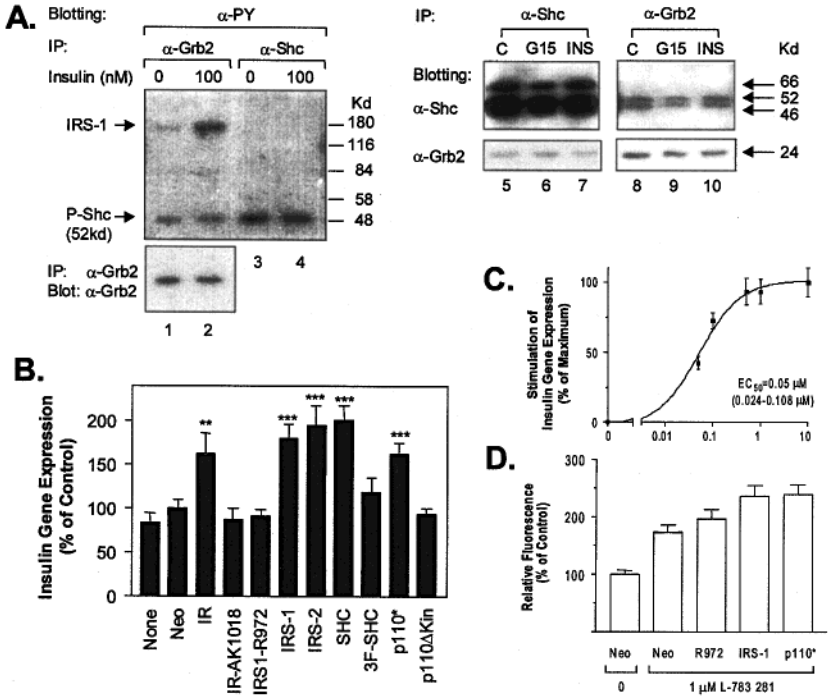

FIGURE 5: Grb2-dependent activation of insulin gene expression. (A) Association of Grb2 and IRS-1, Shc proteins. β-Cell lysates were immunoprecipitated (IP) with anti-Grb2 (α-Grb2, lanes 1, 2, and 8−10) or anti-Shc (α-Shc, lanes 3−7) antibodies and immunoblotted with the indicated antibodies: α-PY, anti-phosphotyrosine antibody (K-18); P-Shc, tyrosine-phosphorylated Shc; C, control; G15, 15 mM glucose; INS, 100 nM insulin. (B) Stimulation of insulin gene expression. βTC6-F7 cells were cotransfected with the RIP-GFP reporter gene and one of the following vectors: None, RIP-GFP only; Neo, Neo vector; IR and IR-AK1018, wild type and the kinase deficient mutant insulin receptor; IRS-1 and IRS1−R972, wild type and the Grb2 interaction deficient mutant IRS-1; Shc and 3F-Shc, wild type and Tyr239Phe, Tyr240Phe, and Tyr317Phe mutant Shc; p110* and p110ΔKin, constitutively active and kinase deficient PI-3 kinase, respectively. Two asterisks indicate that $p < 0.001$ and three asterisks $p < 0.0001$. (C) Dose curve of stimulation of insulin gene expression with L-783 281. EC_{50} is the half-effective concentration. (D) Synergistic effect of L-783 281 and IR signaling molecules: IRS-1, IRS1-R972 (R972), and PI-3K (p110*).

β-cells (comparing the P-Shc bands in lanes 1 and 2 and in lanes 3 and 4).

Using a rat insulin promoter-driven green fluorescence protein (RIP-GFP) reporter gene system, we show that the insulin receptor (IR), IRS-1, IRS-2, and Shc all stimulated insulin gene transcription (Figure 5B). Insulin receptor kinase activity is required for the stimulation since the kinase deficient IR mutant (IR-AK1018) abolished the IR enhancement of insulin gene expression. The interaction with Grb2 is essential for insulin's effect. The mutations that impair Grb2−IRS-1 interaction (IRS1−R972) (*43*) or Grb2−Shc association (3F-Shc, a Shc mutant with Phe replacing Tyr at positions 239, 240, and 317) (*44*, *45*) all failed to stimulate insulin gene expression. Stimulation of insulin gene expression by insulin receptor signaling was glucose-dependent. In the absence of glucose (<0.1 mM), transient overexpression of IR, IRS-1, and IRS-2 all failed to enhance insulin gene expression (data not shown). Using chemical inhibitors, Leibiger and colleagues suggested that PI-3 kinase may be involved in the regulation of insulin gene transcription (*15*, *46*). We demonstrate here that phosphatidylinositol 3-kinase (PI-3K) plays a positive role in insulin gene regulation. The p110* encodes a constitutively active form of p110 (the catalytic subunit of PI-3K) in which the inter-SH2 domain of p85 (the regulatory subunit of PI-3K) was ligated to the NH2 terminus of p110. The p110ΔKin is a kinase deficient version of p110* in which the ATP binding site was mutated (*38*, *39*). In our transient expression system, p110* but not p110ΔKin stimulated insulin gene transcription (Figure 5B). Consistent with the previous report (*46*), rapamycin abolished the stimulatory effect of PI-3K (data not shown). A novel nonpeptidyl fungal metabolite (L-783 281) activates insulin receptor kinase both in vitro and in vivo and has insulin mimetic biological effects (*47*). Activation of the insulin receptor with L-783 281 significantly stimulated insulin gene expression in a dose-dependent manner with a half-effective concentration (EC_{50}) of 0.05 μM (Figure 5C). Combination of L-783 281 and transient overexpression of IRS-1 or p110* had synergistic effects on insulin gene expression (Figure 5D). The mutant IRS-1 (R972) that abolished IRS-1 and Grb2 interaction failed to further enhance L-783 281 stimulation of insulin gene expression. These data demonstrate that insulin receptor signaling positively regulates insulin gene transcription and provide the first evidence that Grb2 plays a key role in regulation of insulin gene expression in β-cells.

DISCUSSION

Our prior studies have shown that in β-cells stably 2-fold overexpressing IRS-1, Ca^{2+} homeostasis was perturbed with an increase in cytosolic [Ca^{2+}] and an increased level of insulin secretion (*11*). We had postulated the existence of a novel autocrine loop of insulin on insulin secretion from the β-cell, mediated by activation of the insulin receptor signaling pathway and IRS-1. In the present study, we have extended these findings to the rat pancreatic islet, a widely accepted model of physiological insulin secretion. Our study shows that insulin treatment of isolated islets results in an increase in islet cytosolic [Ca^{2+}] as well as an increase in the level of insulin secretion. Similar findings have been reported with

isolated mouse islets (*17*), although in that study the effect of insulin was observed within minutes. In addition, studies with a β-cell-specific insulin receptor knockout mouse and IRS-1 deficient β-cell lines show that glucose-induced insulin secretion is lost under those conditions (*16*, *19*). Thus, there is a convergence of data obtained in animal and cellular models demonstrating the existence of a feedback loop of insulin on glucose-induced insulin secretion, which is mediated by an interaction of the insulin signal transduction pathway with the Ca^{2+} homeostatic mechanisms of the β-cell.

Glucose is the main physiological insulin secretagogue (*48*). However, other agents are also important physiologically, most notably, amino acids and depolarizing agents. In β-cells overexpressing IRS-1, both KCl and leucine caused an increase in fractional insulin secretion compared to that in control cells (Figure 3). The amplitude of this increase in the level of insulin secretion was similar to that previously observed in the same cells with glucose (see ref *11*). Furthermore, leucine and KCl treatment also affected β-cell Ca^{2+} homeostasis and increased cytosolic Ca^{2+} levels. Although the peak of increased [Ca^{2+}] induced by KCl was not affected by overexpression of IRS-1, the second phase of cytosolic [Ca^{2+}] was significantly increased, as we had previously observed with glucose, most likely reflecting the inhibition of endoplasmic reticulum Ca^{2+} uptake pump (*11*). In contrast, leucine stimulation of the β-cells overexpressing IRS-1 resulted in higher cytosolic Ca^{2+} levels. Clearly, these results are consistent with our hypothesis that insulin had a positive feedback loop on its own secretion.

IRS-1 overexpression in β-cells specifically induced a 42% inhibition of SERCA3 gene expression (Figure 4), while expression levels of other genes such as IRS-2 and SERCA2 were unchanged. The functional consequence of this decrease in SERCA expression is unclear at the present time, although our previous studies in these cells have demonstrated a decrease in SERCA activity (*11*). However, since both SERCA2b and SERCA3b isoforms are present in β-cells, the contribution of each isoform to the Ca^{2+} uptake activity in the ER remains to be determined. Reduction in the level of SERCA3 gene expression in β-cells has been shown in db/db mice (*31*), in Goto-Kakizaki Wistar (GK) rats (*32*), and also in human diabetes (*33*), but the cause of this reduction remains unknown. IRS-1 inhibition of SERCA3 gene expression in this study reveals a novel regulatory mechanism for β-cell Ca^{2+} regulation. Grb2 may play an important role in mediating this regulation.

This study identifies a novel regulatory pathway of insulin secretion at the molecular level with two main components: (1) regulation of intracellular Ca^{2+} homeostasis and (2) regulation of insulin gene expression. Long-term activation of the insulin receptor signaling system, by either insulin binding to its receptor or amplification of insulin receptor signaling molecules such as IRS-1, induces inhibition of SERCA3 gene expression causing an increase in $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ subsequently stimulates insulin secretion via Ca^{2+}-dependent pathways. Insulin may also directly regulate SERCA3 Ca^{2+}-ATPase activity to increase $[Ca^{2+}]_i$. Recent findings that IRS-1 directly interacts with different ER Ca^{2+}-ATPases, SERCA1 and -2 in muscle and heart cells (*49*), and that IRS-1 and SERCA3 colocalize in β-cell ER membrane (P. D. Borge, data not shown) support this possibility.

Insulin also stimulates insulin gene expression in a Grb2-dependent manner. However, chronic activation of IR signaling reduced the ER Ca^{2+} content through IRS-1 inhibition of ER Ca^{2+} uptake and inhibited insulin biosynthesis at the translational level (*11*). This may explain why, in the long term, insulin fails to enhance net insulin production as described in this study. These data imply that chronic exposure to insulin may eventually exhaust β-cell insulin storage. Long-term exposure to stimulatory concentrations of glucose, e.g., 20 mM, has a dual effect on β-cells. It promotes the release of insulin, which initially further stimulates insulin secretion and affects other β-cell function as described above. Due to the constant stimulation of insulin release, although glucose also enhances insulin gene expression and insulin biosynthesis, the net effect of long-term exposure to a high level of glucose has been shown to reduce the islet intracellular insulin storage capacity (Figure 2C). These results indicate that hyperinsulinemia and hyperglycemia, both characteristic of type 2 diabetes, may over time have a negative impact on β-cell function.

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